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13. ABSTRACT (Maximum 200 Words)

To define the causative role of the active Akt in androgen-independent progression of prostate cancer, an inducible Akt (iAKT) is used in this proposal. In this second-year period, androgen-dependent human prostate cancer LNCaP cells stably transfected with vectors carrying iAKT system or the control vectors (empty and kinase-dead mutant iAKTkm) were used to determine if active Akt can protect cell death or promote cell proliferation after serum starvation. We demonstrated that CID-mediated activation of iAKT promotes cell survival only but not cell proliferation of prostate cancer LNCaP cells after androgen or serum withdrawal. We also demonstrated that the established LNCaP.iAKT cells formed xenograft in nude mice and CID AP22783 injection showed a promising ability to activate the iAKT, and the activated iAKT is functionally capable to phosphorylate the downstream target GSK-3. In the next year, we will use those LNCaP subline cells to determine whether CID-mediated iAKT activation promote tumor growth in castrated nude mice.

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Annual Report for the Project DAMD17-03-1-0121

February 2005

Project Title:

The Role of Akt in Androgen-independent Progression of Human Prostate Cancer

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Introduction

Prostate cancer is the second most frequently diagnosed cancer in men after skin cancer in United States (1). While digital rectal exams and early prostate specific antigen screening have led to earlier detection and diagnosis, the number of new cases continues to rise and present a major worldwide health threat. Medical treatment for metastatic prostate cancer has relied heavily on androgen ablation (2). However, there has been a growing appreciation that most patients treated by androgen ablation ultimately relapse to more aggressive androgen-independent (also called hormone-refractory) prostate cancer with no means to cure (3). Although Akt has been shown to be associated with prostate cancer progression (4-9), determining the causative role of this specific kinase in initiating or maintaining the androgen-independent progression of prostate cancer has not been established. One major limitation is the lack of a well-controlled inducible system to study Akt involvement in prostate cancer. Recently, we have developed a novel inducible Akt (iAKT) system based on chemically induced dimerization (CID) approach. This system allows for selective activation of Akt in a physiological setting. This CID-mediated activation of iAKT is the major innovative feature of this proposal (***Appendix I***). We propose that active Akt plays a causative role in androgen-independent progression of human prostate cancer via protecting cell death and enhancing cell proliferation after androgen withdrawal. ***Initially,*** androgen-dependent human prostate cancer LNCaP cells will be transfected with vectors carrying iAKT system or control vectors (empty and kinase-dead mutant iAKTkm) to establish stable sublines. ***Next,*** the sublines will be used to determine whether CID-mediated activation of iAKT can lead to survival or proliferation after androgen withdrawal. ***Finally,*** LNCaP tumor will be established subcutaneously in male nude mice by inoculating LNCaP subline cells stably expressing iAKT or kinase-dead mutant iAKTkm. After castration or sham-operation, the animals will receive AP22783 injection to induce Akt activation. Tumor growth will be monitored to determine if CID-mediated activation of iAKT promotes androgen-independent LNCaP tumor growth.

Last year, we reported that several LNCaP cell sublines were established to ectopically express the iAKT system and the CID-induced Akt activation was determined.

Report Body

In this section, we will describe the current status and results obtained from the experiments outlined in the approved Statement of Work, which is listed below:

Task 1. c. Determine whether CID-mediated activation of iAKT promotes cellular survival or proliferation in LNCaP subline cells after androgen withdrawal (Months 13-18)

As mentioned earlier, we already established several clones that express the iAKT system, which can be activated by the CID in the subline cells. Then we went on to test if CID-mediated Akt activation will protect those cells from apoptosis after serum starvation or promote cell proliferation after androgen-withdrawal. LNCaP.iAKT, LNCaP.AKTkm or LNCaP.Neo cells were used and cell growth/death rate was measured using trypan blue exclusion assay. Apoptotic event was determined using Annexin V-FITC labeling method on FACS, and cell proliferation was determined by BrdU incorporation.

Briefly, cells were serum-starved for 24 h and then treated with or without CID AP22783 (200 nM) in serum-free media in cell death experiments or in 2% charcoal-stripped fetal bovine serum (cFBS) in cell proliferation experiments. Cells were monitored for up to a week. Cells grown in full media without any treatment were used as a positive control. After treatment, cells were washed with ice-cold PBS and then subjected to the assays as mentioned above. Cell proliferation rate for all three cell lines cultured in 2% cFBS or AP22783 condition was significantly reduced compared to that in full media (10% FBS), as measured in BrdU incorporation assay (Fig 1) and trypan blue exclusion assay (data not shown). When LNCaP.iAKT cells were treated with CID AP22783, no significant enhancement in term of cell proliferation was observed compared to no treatment control, LNCaP.iAKTkm or LNCaP.Neo cells treated with CID AP22783 (Fig 1). However, AP22783 addition dramatically reduced serum starvation-induced apoptotic cell death in LNCaP.iAKT cells compared to LNCaP.iAKTkm or LNCaP.Neo cells. CID-mediated iAKT activation did not restore a full survival rate when compared to the full-serum cultures. These results indicate that CID-mediated active iAKT protects androgen-dependent LNCaP cells from serum withdraw-induced cell death but does not enhance cell proliferation.

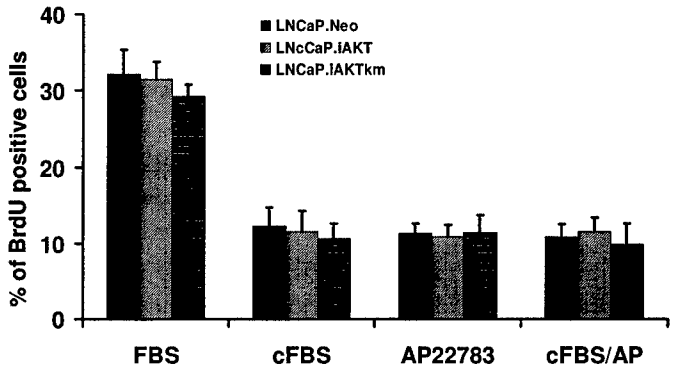

Fig 1. BrdU incorporation assay for cell proliferation. Cells were seeded in 12-well plates and serum-starved overnight. Cells were treated as indicated (FBS 10%; cFBS 2%; AP22783 200 nM; & cFBS plus AP22783) for 24 h and then subjected to Flow Cytometry-based BrdU incorporation assay.

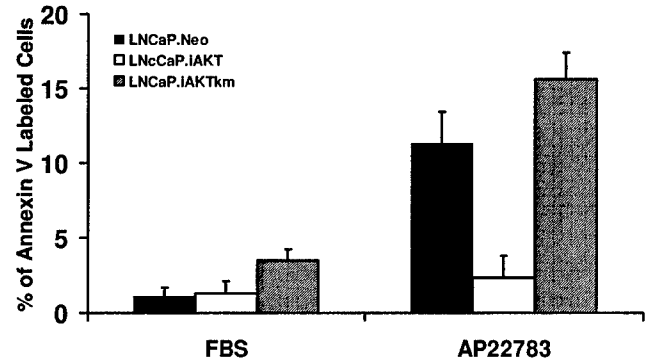

Fig 2. Annexin V labeling assay for apoptotic cell death. Cells were seeded in 6-well plates and serum-starved for 24 h before adding FBS back or treated with AP22783 (200 nM). Cells were harvested 7 days later and subjected to FCM-based Annexin V labeling assay.

Task 2. Determine whether CID-mediated activation of iAKT promotes LNCaP tumor growth in castrated nude mouse (Months 18-36).

a. Pilot study on 4 nude mice to clarify CID-mediated iAKT activation in LNCaP tumors *in vivo*. (Months 18-20)

Next, we performed a pilot study to clarify CID AP22783-mediated iAKT activation in LNCaP xenograft. LNCaP xenografts were developed in 4 six-week old athymic male mice using the LNCaP.iAKT sublines as described earlier. Four weeks later when tumors became palpable, animals were received a single dose of 2 mg/kg AP22783 injection or carrier control (2 mice per each treatment). Xenografts were harvested 12 h later and protein extracts were prepared as described in our previous publication (3). Phosphorylation and activity status of the iAKT were measured by Western blot and an in vitro kinase assay. As shown in Fig. 3, CID AP22783 injection dramatically induced Akt S473 phosphorylation and kinase activity toward to GSK-3 phosphorylation. Currently, we are performing a full-scaled animal experiment to determine if CID-mediated activation of iAKT promotes androgen-independent LNCaP tumor growth.

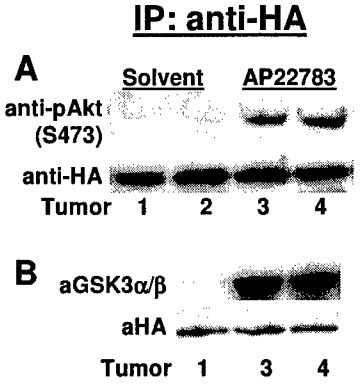

Fig 3. CID-mediated iAKT activation *in vivo*. Protein extracts were prepared from LNCaP.iAKT xenografts after solvent (tumor 1&2) or AP22783 (tumor 3&4) treatment. The iAKT was immnuoprecipitated using anti-HA antibody. The phosphorylation status of S473 was determined using a specific antibody. Anti-HA blot served as a loading control. Akt kinase activity assay was done as described in Ref. 10

Key Research Accomplishments

For the second year of this project according to the proved State of Work, we accomplished:

1. Demonstrated that CID AP22783-mediated Akt activation protects cells from serum starvation-induced apoptotic cell death but no enhancing effect on cell proliferation;
2. Demonstrated that CID AP22783 can activate the iAKT system when the LNCaP subline cells carrying the system are allocated into nude mice, as evidenced by iAKT phosphorylation (pAkt S^{473}) and activation (kinase activity towards to GSK-$3\alpha^{21}/\beta^{9}$ phosphorylation).
3. Established few stable LAPC-4 (11) sublines bearing the empty vector only or the iAKT system for further experiments in cell proliferation because no such effect was observed in LNCaP-derived cell sublines;
4. Also established few stable RWPE-1 (12) bearing the empty vector only or the iAKT system for further experiments in cell proliferation because no such effect was observed in LNCaP-derived cell sublines;

Reportable Outcomes

LAPC-4 sublines:

3 clones of LAPC4.iAKT cell lines;
2 clones of LAPC4.Neo cell lines;

RWPE-1 sublines:
2 clones of RWPE1.iAKT cell lines;
2 clones of RWPE1.Neo cell lines.

Conclusion

In this second year period, we continued previous work and completed the proposed experiemnts according to the State of Work (month 13-20). Using the LNCaP sublines expressing the iAKT system construct as well as the control vectors, we demonstrated that CID AP22783-induced iAKT activation reduced serum starvation-triggered apoptotic cell death, but no effect was observed on cell proliferation. Also, when the established LNCaP.iAKT cells were injected into nude mice, tumor formation was accomplished and injection of the CID led to iAKT activation. Currently, we are in the middle of conducting the **Task 2B&C** in a large group to determine if AP22783-activated iAKT promotes tumor growth in castrated nude mice.

In addition to the proposed work, we established more stable cell lines that express the iAKT or a control construct in two other prostate-derived cell lines, LAPC-4 and RWPE-1. The rationale is that cell proliferation was not enhanced by iAKT activation in LNCaP-derived cell sublines, and LAPC-4 and RWPE-1 cells are prostate-derived AR positive cell lines that express a wild type AR and PTEN, both molecules are mutant in LNCaP cells and are critical for cell fate determination. We will use these sublines to rule out the cell-based differences regarding AKT's role in cell proliferation.

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10. **Benyi Li,** Desai, S.A., MacCorkle-Chosnek, R.A., Fan, L. and Spencer, D.M. A novel conditional Akt 'survival switch' reversibly protects cells from apoptosis. ***Gene Ther*** 2002; 9(4):233-244.

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Appendix

A previous publication describing the creation of the iAKT system is attached to this report.

1. **Benyi Li,** Desai, S.A., MacCorkle-Chosnek, R.A., Fan, L. and Spencer, D.M. A novel conditional Akt 'survival switch' reversibly protects cells from apoptosis. ***Gene Ther*** 2002; 9(4):233-244.

Gene Therapy (2002) 9, 233–244

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RESEARCH ARTICLE

A novel conditional Akt 'survival switch' reversibly protects cells from apoptosis

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The anti-apoptotic Akt kinase is commonly activated by survival factors following plasma membrane relocalization attributable to the interaction of its pleckstrin homology (PH) domain with phosphatidylinositol 3-kinase (PI3K)-generated PI3,4-P_2 and PI3,4,5-P_3. Once activated, Akt can prevent or delay apoptosis by phosphorylation-dependent inhibition or activation of multiple signaling molecules involved in apoptosis, such as BAD, caspase-9, GSK3, and NF-κB and forkhead family transcription factors. Here, we describe and characterize a novel, conditional Akt controlled by chemically induced dimerization (CID). In this approach, the Akt PH domain has been replaced with the rapamycin (and FK506)-binding domain, FKBP12, to make F3-ΔPH.Akt. To effect membrane recruitment, a myristoylated rapamycin-binding domain from FRAP/mTOR, called M-FRB, binds to lipid permeable rapamycin (and non-bioactive synthetic 'rapalogs'), leading to reversible heterodimerization of M-FRB with FKBP-ΔPH.Akt. Like endogenous c-Akt, we show that the kinase activity of membrane-localized F3-ΔPH.Akt correlates strongly with phosphorylation at T308 and S473; however, unlike c-Akt, phosphorylation and activation of inducible Akt (iAkt) is largely PI3K independent. CID-mediated activation of iAkt results in phosphorylation of GSK3, and contributes to NF-κB activation in vivo in a dose-sensitive manner. Finally, in Jurkat T cells stably expressing iAkt, CID-induced Akt activation rescued cells from apoptosis triggered by multiple apoptotic stimuli, including staurosporine, anti-Fas antibodies, PI3K inhibitors and the DNA damaging agent, etoposide. This novel inducible Akt should be useful for identifying new Akt substrates and for reversibly protecting tissue from apoptosis due to ischemic injury or immunological attack.
Gene Therapy (2002) **9**, 233–244. DOI: 10.1038/sj/gt/3301641

Keywords: *Akt; CID; apoptosis; survival switch; conditional signaling protein; inducible Akt*

Introduction

When growth factors are limiting in the extracellular milieu, most cell types die by apoptosis due to finely tuned homeostatic mechanisms. One common pathway by which ligand-bound growth factor receptors prevent apoptosis is through the phosphorylation-dependent membrane recruitment and activation of phosphatidylinositol 3-kinases (PI3K). PI3Ks generate phosphatidylinositol 3,4-diphosphate (PtdIns(3,4)P_2) and PtdIns(3,4,5)P_3 by phosphorylating the D-3 position of the inositol ring of phosphoinositides. In turn, these 3-phosphorylated lipids can lead to the plasma membrane recruitment and activation of a number of cytosolic signaling molecules by binding to their pleckstrin homology (PH) domains. The importance of the PH domain is underlined by its recent discovery in over 250 genes, the 11th most common InterPro family found in the human proteome.[1] Although the cellular responses regulated by PI3Ks are diverse, including growth, survival, transformation, vesicle trafficking, and others (reviewed in Ref. 2), activation of the serine/threonine kinase Akt/PKB (the cellular homologue of the viral oncogene, v-Akt), appears to be central to the PI3K-mediated delay of apoptosis and increase of cell survival.[3]

Although c-Akt was cloned a decade ago,[4] the mechanism by which Akt propagates survival signals in eukaryotic cells has only been elucidated more recently (reviewed in Ref. 5). All three mammalian isoforms of Akt (Akt1/PKBα/RAC-PKα, Akt2/PKBβ/RAC-PKβ, and Akt3/PKBγ/RAC-PKγ) have an amino-terminal PH domain, a serine-threonine (S/T) kinase domain related to protein kinase A and C (PKA and PKC) family members, and a carboxy-terminal regulatory domain. Akt is activated in response to various survival stimuli, such as growth factors, cytokines and hormones, in a PI3K-dependent manner.[6] In addition, PI3K-independent activation of Akt has also been shown after treatment with heat shock,[7] β-adrenergic receptor activation,[8] PKC activation,[9] and c-AMP up-regulation.[10] It is believed that Akt activation involves three steps, in which the first step is the interaction of the inhibitory PH domain with PtdIns(3,4)P_2 and PtdIns(3,4,5)P_3 leading to membrane recruitment and a conformational change in the kinase. Together these two events expose T308 (based on Akt1) in the activation loop of the catalytic domain to the constitutively active, PtdIns(3,4,5)P_3-dependent kinase-1 (PDK1). Finally, T308 phosphorylation leads to phosphorylation in the regulatory domain at S473 (Akt1) by PDK2. Although PDK2 is still poorly defined, PKC members, integrin-linked kinase (ILK), PDK1 (bound to

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PRK2[11]), and Akt autophosphorylation[12] have all been reported to be the effectors of this event (reviewed in Refs 3 and 5).

Further underlying the nodal position of Akt in survival signaling are the observations that pT308 and pS473 have a relatively short half-life *in vivo*, and phosphatase inhibitors, such as calyculin A and okadaic acid, a relatively specific inhibitor of PP2A, are able to prevent Akt dephosphorylation and inactivation.[13] Moreover, Akt family members are up-regulated in several cancers and inactivation of the PtdIns phosphatase, PTEN, is also associated with cancer and Akt activation.[14–19]

To date, Akt has been implicated in various physiological processes including cell cycle regulation, cellular metabolism and cell survival. The first identified downstream target of Akt was glycogen synthase kinase-3 (GSK-3), which is phosphorylated at serine 21 in GSK3-α and serine 9 in GSK3-β, leading to inactivation and the up-regulation of a number of substrates involved in cellular metabolism, including glycogen synthesis.[20] Recently, several targets of the PI3K/Akt signaling pathway have been identified that may explain the ability of this regulatory cascade to promote survival (reviewed in Ref. 5). These targets include two components of the intrinsic cell death machinery, Bad and caspase 9, transcription factors of the forkhead family (ie AFX) that can up-regulate FasL, and the kinase, IKKα, that regulates the anti-apoptotic transcription factor, NF-κB. Additional substrates for Akt include eNOS, phosphofructokinase-2, phosphodiesterase 3β and the reverse transcriptase subunit of telomerase. These and other as yet unidentified Akt substrates might mediate the effects of Akt on cellular survival.

In order to elucidate the function of many signaling molecules, constitutively active or 'dominant negative' mutant proteins are often overexpressed in target cells. When Akt or many other 'upstream' signaling molecules are modified to contain a membrane targeting sequence, the increased proximity to activating kinases, such as PDK1, or to membrane-localized substrates typically leads to the constitutive phenotype. For example, most functional Akt studies have utilized either Src family myristoylation-targeting peptides or the myristoylated gag sequence within v-Akt. Under these conditions, however, the kinase is activated as soon as it is expressed in cells, but the effects of activation may not be monitored until much later, when the direct effects of Akt are typically obscured.

For controlled gene expression or kinase activation, several approaches are available such as tetracycline-regulatable transcription systems,[21] chimeras of hormone binding domains (HBD) with target proteins[22–24] and chemically induced dimerization (CID).[25,26] The CID system uses synthetic bivalent ligands to rapidly crosslink signaling molecules that are fused to ligand-binding domains

CID has been used to trigger the oligomerization and activation of cell surface,[25,27,28] or cytosolic proteins,[29,30] the recruitment of transcription factors to DNA elements to modulate transcription,[31,32] or the recruitment of signaling molecules to the plasma membrane to simulate signaling.[33,34] Here, we extend these applications with the development of a highly responsive, conditional Akt molecule, called inducible Akt (iAkt) whose range of activation extends from undetectable to comparable to that of constitutively active Myr-Akt. Activation of iAkt is based on ligand-dependent recruitment of chimeric Akt to a membrane-bound myristoylated 'docking protein'. To improve the CID-responsiveness of iAkt, we utilized novel, high specificity heterodimerizing ligands, called rapalogs, based on the immunosuppressant drug, rapamycin.[35] Otherwise bioinert, rapalogs can reversibly crosslink proteins fused to the proline isomerase, FKBP12, with FRB_1 a 90 amino acid rapamycin-binding domain excised from the S6 kinase kinase, FRAP/mTOR, containing L2098 to confer specificity. While rapalogs bind with high affinity to FRB_1 binding to endogenous FRAP is sterically prevented. Thus, Akt fused to FKBPs can be efficiently recruited to the plasma membrane and activated.

Although iAkt based on full-length Akt, had very high basal activity due presumably to high CID-independent membrane association, removal of the PH domain in iAkt variant, F3-ΔPH.Akt eliminated basal activity while concomitantly permitting CID dose-dependent phosphorylation at T308 and S473 and Akt activation. Further, we show that CID-dependent Akt activation is largely PI3K independent, can greatly augment NF-κB activity and can lead to phosphorylation of GSK3α/β. In turn, conditional activation of iAkt leads to reversible protection from a number of apoptotic stimuli, including the PI3K inhibitors wortmannin and LY294002, the broad-spectrum protein kinase inhibitor, staurosporine, DNA damage mediated by the topoisomerase II inhibitor, etoposide, and Fas crosslinking. As gene therapy comes of age the ability to conditionally regulate viability with an anti-apoptotic 'survival switch' like iAkt is likely to be as useful as the more well-established pro-apoptotic suicide genes.

Results

CID-mediated activation of Akt/PKB

The strategy used in this study to develop conditional alleles of Akt kinase is based on the knowledge that membrane targeting of endogenous Akt family members (ie AKT1, 2, 3) leads to their phosphorylation and activation.[36] Similarly, several groups have shown that myristoylated (M) Akt (ie M-Akt) leads to its constitutive activation (reviewed in Ref. 5). Therefore, we used conditionally induced dimerization (CID) to reversibly recruit Akt1 to the plasma membrane. It was shown previously that rapamycin could crosslink endogenous FKBP12 with a 90 amino acid domain from FRAP/mTOR, called FRB (FRAP rapamycin binding domain, residues 2025–2113).[37,38] More recently, 'rapalogs' have been developed that are modified to greatly reduce binding, and therefore bioactivity, to endogenous FRAP, but are able to bind with high affinity to modified FRB_1 (T2098L).[35] As shown in Figure 1a, the heterodimeric rapalog/CID_{HED} can effect the crosslinking of FRB_1 and FKPB12 (called F). In these experiments, we used the non-toxic variant of FKBP12, F_{pk} (FKBP12(G89P,190K)), to eliminate background toxicity.[30] For the plasma membrane docking protein, we fused a c-Src myristoylation signal to one or two copies of FRB_1, to create M-FRB_1 and M-$FRB_1$2, respectively (Figure 1b). To create inducible Akt (iAkt) molecules, three tandem FKBP domains (F3) were fused to the N- or C-termini of wild-type Akt or a variant (ΔPH.Akt), lacking the pleckstrin homology (PH)

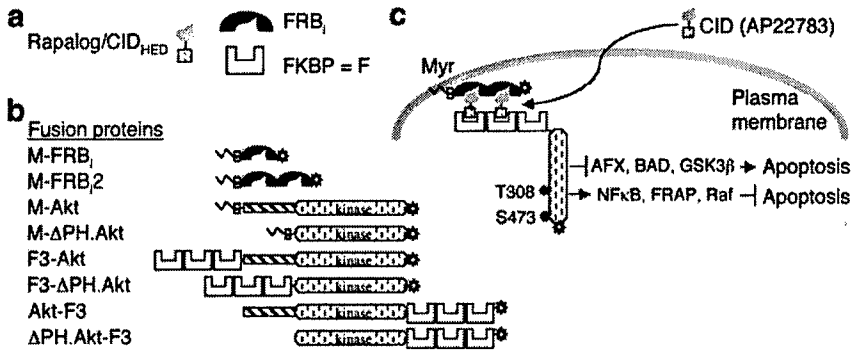

Figure 1 Schematic representation of constructs used in this study. (a) Heterodimeric (HED) CIDs/rapalogs and CID_{HED}-binding domains. Synthetic ligand, AP22783, is a bivalent compound that binds to the FRB_1 domain (but not endogenous FRAP/mTOR) and concomittantly to FKBP12 (F) variants. (b) The CID-binding domains were subcloned as monomers (FRB_1), dimers ($FRB_1$2), or tandem trimers (F3) into expression vectors to generate chimeric proteins. The c-Src myristoylation (M) signal sequence (horizontal bars) was fused to the N-terminus of FRB_1 or Akt kinase alleles (vertical dashes). Wild-type or PH domain (striped) deletion mutants of Akt were fused to F3 at their N- or C-terminal ends. All constructs used in this study have an HA epitope tag (star) at their C-terminus. (c) Model of CID_{HED}-mediated membrane targeting and activation of inducible Akt (iAkt) kinase. Cell permeable rapalogs (eg AP22783) bring F3-ΔPH.Akt to the plasma membrane by crosslinking FRB_1 and FKBP domains, triggering Akt phosphorylation at two key residues for activation, T308 and S473. Phosphorylation of multiple downstream substrates can rescue cells from death by inhibition of promoters of apoptosis or activation of inhibitors of apoptosis.

domain to reduce natural membrane association. Furthermore, we developed constitutively active, myristoylated Akt (M-Akt) or M-ΔPH.Akt and kinase-dead mutant versions (ie Akt.K179M, named AktKM) of chimeric Akt constructs. For identification and purification, all chimeric constructs were HA epitope-tagged (name left off for simplicity). Thus, membrane recruitment of F3-modified Akt by rapalogs, such as AP22783 used in these experiments, leads to phosphorylation of Akt at T308 and S473, induction of Akt kinase activity, and phosphorylation of downstream effector molecules, leading to modulation of apoptotic signals.

Membrane targeting of PH domain-less Akt leads to rapalog dose-dependent activation of NF-κB

Two key requirements for efficient synthetic regulation of a biological event are highly specific conditional dependency and low background. NF-κB induction is a major target of Akt following growth factor signaling, and multiple reports show that a constitutively active myristoylated Akt (M-Akt) can enhance protein kinase C (PKC)-mediated NF-κB induction by either phosphorylation of IKKα,[39] the activation domain of p65/ReIA,[40] or both.[41] Therefore, in order to optimize iAkt, we hypothesized that the ability of iAkt variants to complement PKC-induced NF-κB transcriptional activity using an NF-κB-responsive secreted alkaline phosphatase (SEAP) reporter plasmid would be a sensitive assay for Akt activation.

To validate this assay, the human T cell line, Jurkat-TAg, was cotransfected with reporter plasmid, NF-κB/SEAP, along with constitutively active M-Akt expression vector or empty control vector. Twenty-four hours after transfection, cells were divided into aliquots that were stimulated with sub-optimal levels (5 ng/ml) of the phorbol ester, PMA, or were untreated. After an additional 24 h, SEAP activity was measured. Although Akt activity alone was insufficient to induce measurable NF-κB activity, M-Akt expression could greatly potentiate (by three-to four-fold) PKC-induced NF-κB activity, consistent with multiple reports. Furthermore, inhibition of PI3K by LY294002 (5 μM) or wortmannin (1 μM) did not prevent NF-κB activation by M-Akt plus PKC, although inhibition of 'typical' PKC isoforms with R0318220 (1 μM) led to complete inhibition of NF-κB as expected (Figure 2).

Since the constitutively active Akt (T308) kinase, PDK1, is primarily membrane-associated following growth factor stimulation, membrane recruitment of Akt via its PH domain is necessary for its activation.[42–44] Furthermore, although the PH domain has been shown to suppress basal phosphorylation of T308 and Akt activation when not bound by its lipid ligand, PIP_2, this initial phosphorylation should still require interaction with membrane-localized PDK1.[45] Therefore, we reasoned that removing the PH domain in iAkt should theoretically lead to low basal activity and a large activation index. To test this hypothesis, we compared basal Akt activity and activation following membrane recruitment of full-length

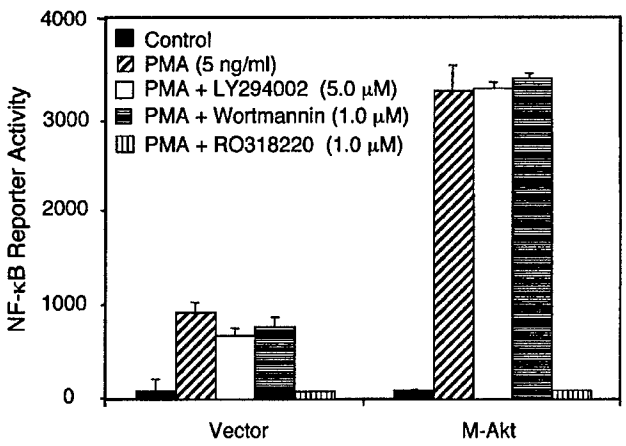

Figure 2 M-Akt enhances NF-κB induction induced by PMA. Jurkat-TAg cells were cotransfected with NF-κB/SEAP reporter plasmid along with control vector or M-Akt expression vector by electroporation. After 24 h, cells were treated with sub-maximal levels (5 ng/ml) of PMA plus PI3K inhibitor, LY294002 (5 μM) or wortmannin (1 μM), PKC inhibitor, RO318220, (1 μM), or control diluent. SEAP activity was measured 24 h later as described in Materials and methods. Data shown are the mean and s.d. of two duplicate experiments.

and truncated Akt, lacking the PH domain, in our NF-κB reporter assay.

We fused both full-length and ΔPH.Akt to a tandem trimer of the CID-binding domain, F3, at both the amino and carboxyl termini. As before, Jurkat-TAg cells were cotransfected with reporter plasmid NF-κB/SEAP along with the membrane docking molecule, M-$FRB_1$2, alone, various F3-Akt chimeras, alone, or both together. Twenty-four hours after transfection, cells were stimulated with 5 ng/ml PMA along with log dilutions of heterodimerizing CID, AP22783. After additional 24 h incubation, SEAP activity was assayed. As shown in Figure 3a, wild-type Akt showed significant CID-independent NF-κB induction that was only slightly increased by crosslinking to the membrane, via M-$FRB_1$2. This was true regardless of whether F3 was fused to the N- or C-terminus (not shown) of Akt. As expected, membrane recruitment or overexpression of kinase-deficient Akt.KM (K179M) had no detectable effect on NF-κB induction over PMA alone. Thus, membrane recruitment of full-length Akt only slightly increases its activity due to the high basal activity from its overexpression.

In contrast, membrane recruitment of F3-ΔPH.Akt showed a very clear CID-dependent induction of NF-κB with undetectable CID-independent activity (Figure 3b). Moreover, myristoylated M-ΔPH.Akt was more active than M-Akt in augmenting NF-κB activation, consistent with an inhibitory function for the PH domain. Again, M-$FRB_1$2 alone or recruitment of kinase dead F3-ΔPH.AktKM did not influence NF-κB induction (Figure 3b and not shown). These results indicate that the chimeric F3-ΔPH.Akt allele is strongly CID-inducible with very low basal activity. Also, these results are consistent with previous reports that the PH domain of Akt kinase is responsible for its translocation to the plasma membrane and also has an inhibitory function.

Since most applications of CID technology have been based partly, at least, on empirically designed inducible chimeric proteins, CID-mediated targeting or crosslinking might not always faithfully reflect physiological signaling. Further, CID-binding domains, like FKBP12, could potentially sterically hinder an essential target protein domain(s). Therefore, we tested iΔPH.Akt with F3 fused to both termini of ΔPH.Akt. As shown in Figure 4a, the N-terminal fusion chimera, F3-ΔPH.Akt, potentiated NF-κB transactivation somewhat better than the C-terminal chimera, ΔPH.Akt-F3. Since both molecules were expressed at similar levels (not shown), membrane recruitment of F3-ΔPH.Akt may place Akt in a more favorable orientation for interacting with PDK1 or other interacting proteins. In either orientation, however, both iAkt versions were devoid of detectable basal NF-κB signaling.

Since M-$FRB_1$2 could potentially recruit two chimeric Akt molecules simultaneously, we wanted to determine if membrane recruitment of one Akt molecule was sufficient for optimal activation or whether oligomerization of multiple Akt molecules might enhance activation. Therefore, we compared CID-mediated iAkt activity when the membrane docking molecule, contained one or two FRB_1 domains (FRB_1 *versus* $FRB_1$2, respectively). As shown in Figure 4b, there was no significant difference in NF-κB induction by iAkt whether one or two tandem FRB_1 domains were used for the docking site, indicating that forced Akt oligomerization is not a prerequisite for its activation. Nevertheless, this experiment does not rule out that Akt crosslinking might contribute to its activation under normal circumstances.

CID-dependent membrane targeting of Akt kinase results in rapid phosphorylation and activation

Following membrane targeting of endogenous Akt-1, phosphorylation at two highly conserved residues, T308 in the activation loop, and S473 in the inhibitory domain, occurs by PDK1 and PDK2, respectively.[46] To directly address whether CID-mediated membrane targeting of iAkt also leads to phosphorylation at those sites, 293T cells were transiently co-transfected with M-$FRB_1$2 and F3-ΔPH.Akt expression plasmids. After 24 h, transfected cells were serum starved for another 24 h and then

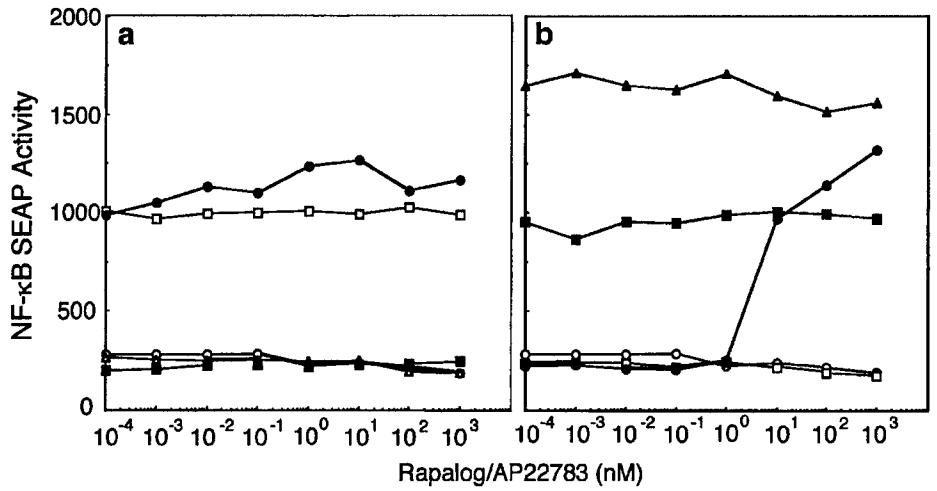

Figure 3 CID-mediated membrane targeting of ΔPH.Akt, but not wild-type Akt, induces titratable NF-κB transactivation. (a and b), Jurkat-TAg cells were transiently cotransfected with reporter plasmid NF-κB/SEAP along with (a) M-$FRB_1$2 (open circle), F3-Akt (open square), F3-Akt.KM (triangle), M-$FRB_1$2 + F3-Akt (closed circle), M-$FRB_1$2 + F3-Akt.KM (closed square), or (b) M-$FRB_1$2 (open circle), F3-ΔPH.Akt (open square), M-$FRB_1$2 + F3-ΔPH.Akt (closed circle), M-Akt (closed square), or M-ΔPH.Akt (triangle). After 24 h, cell aliquots were treated with PMA (5 ng/ml) plus half-log dilutions of the rapalog, AP22783.SEAP activity was measured 24 h later and reported directly. Data are representative of at least two experiments.

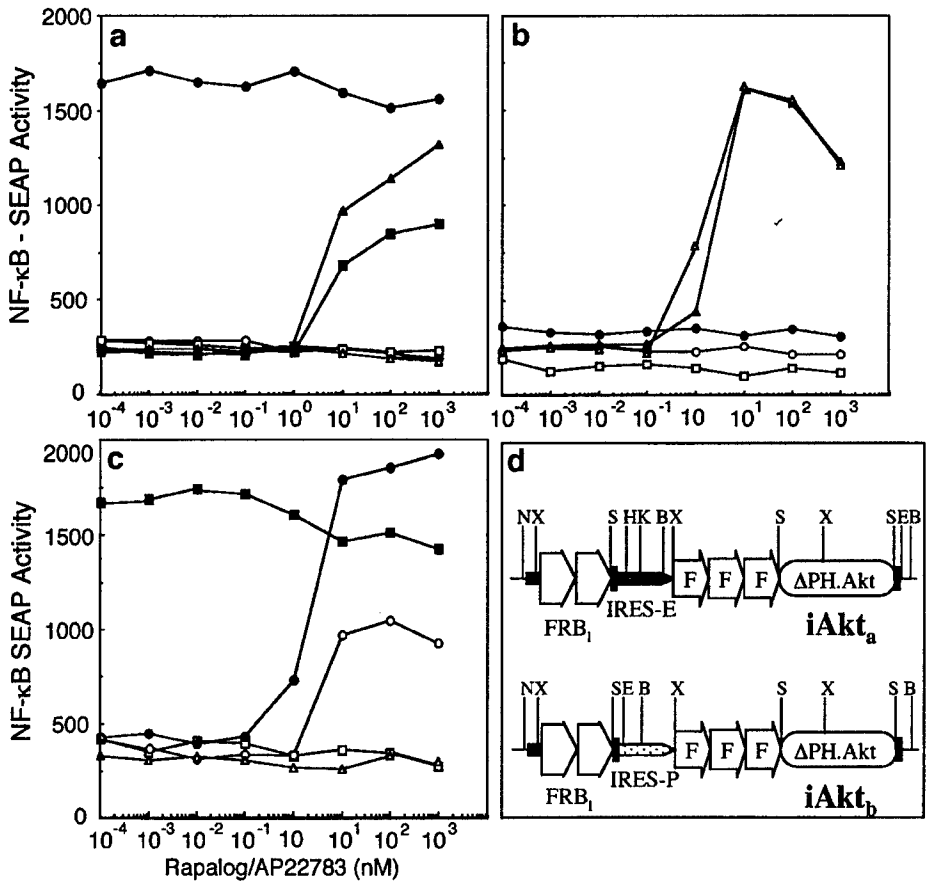

Figure 4 Optimization of iAkt based on CID-mediated NF-κB induction. (a–c) Jurkat-TAg cells were transiently cotransfected with NF-κB/SEAP along with (a) M-FRB$_1$2 (open circle), ΔPH.Akt-F3 (open square), F3-ΔPH.Akt (open triangle), M-FRB$_1$2 + ΔPH.Akt-F3 (closed square), M-FRB$_1$2 + F3-ΔPH.Akt (closed triangle), M-ΔPH.Akt (closed circle), or (b) F3-ΔPH.Akt (closed circle), M-FRB$_1$2 (open circle), M-FRB$_1$ (square), M-FRB$_1$2 + F3-ΔPH.Akt (closed triangle), M-FRB$_1$ + F3-ΔPH.Akt (open triangle), or (c) M-FRB$_1$2 (triangle), F3-ΔPH.Akt (open square), iAkt$_a$ (open circle), iAkt$_b$ (closed circle), or M-ΔPH.Akt (closed square), and treated as above. (a) The orientation of Akt and FKBP has only a minor influence on efficacy. (b) Membrane recruitment of iAkt without oligomerization is sufficient for activation. (c) Bicistronic vector, iAkt$_b$, containing M-FRB$_1$2 and F3-ΔPH.Akt separated by the poliovirus IRES, functions more efficiently than iAkt$_a$ that uses the EMCV IRES. For each experiment, data are representative of three individual experiments. (d) Schematic (scaled) of iAkt$_a$ and iAkt$_b$. M-FRB$_1$2-E and F3-ΔPH.Akt are separated by EMCV (E) or polio (P) IRES sequences, respectively, in expression vector pSH1 (not shown). Myristoylation (M), horizontal black bar. HA epitope (E), vertical black bar. N, NcoI; X, XhoI; S, SalI; H, HindIII; K, KpnI; B, BamHI; E, EcoR1.

treated with AP22783. As shown in Figure 5a, AP22783 treatment greatly stimulated interaction with phospho-specific antibodies against Akt S473 and T308 sites as early as 30 min after drug addition. Although 120 min serum (10% FBS) treatment stimulated phosphorylation of the endogenous protein, serum did not stimulate phosphorylation of iAkt during this period. Curiously, activation of iAkt led to increased phosphorylation of endogenous Akt, particularly at S473. This is not likely to be an effect of AP22783, since this does not happen in control-transfected cells. Therefore, it is likely that activation of iAkt can lead to partial activation of endogenous Akt. These results demonstrate that CID-mediated membrane targeting of iAkt chimeras stimulates Akt phosphorylation.

For targeting tissues or cell lines with bigenic inducible proteins, multi-cistronic vectors can be used to ensure that two, or more, proteins are coexpressed; however, the efficiency of distinct internal ribosome entry sequences (IRES) can be tissue-specific. Therefore, we developed two different bicistronic iAkt vectors, using either the commonly used EMCV IRES, called iAkt$_a$, or iAkt$_b$, using the less characterized IRES from poliovirus. Following electroporation of bicistronic vectors into Jurkat cells and AP22783 stimulation, we observed consistently higher NF-κB induction by iAkt$_b$ compared with iAkt$_a$ (Figure 4c). Therefore, iAkt$_b$ was used to create variant Jurkat lines stably expressing both FRB$_1$2 and F3-ΔPH.Akt, called Jurkat.iAkt cells.

To further measure the induction of Akt enzymatic activity, Jurkat.iAkt cells were serum starved for 24 h and thereafter treated with AP22783 or serum for 30 min. Chimeric F3-ΔPH.Akt kinase was immunoprecipitated with the anti-HA antibody and Akt kinase activity was measured using an *in vitro* kinase assay that uses a GSK3α/β 'crosstide' as a substrate. The level of phosphorylated GSK3 crosstides was determined by immunoblotting with phospho-specific antibody against GSK3-α21/β9. As shown in Figure 5b, only AP22783 treatment, but not serum or mock treatment, was associated with GSK3 phosphorylation in this assay, indicating, as above, Akt activation after CID-mediated membrane targeting.

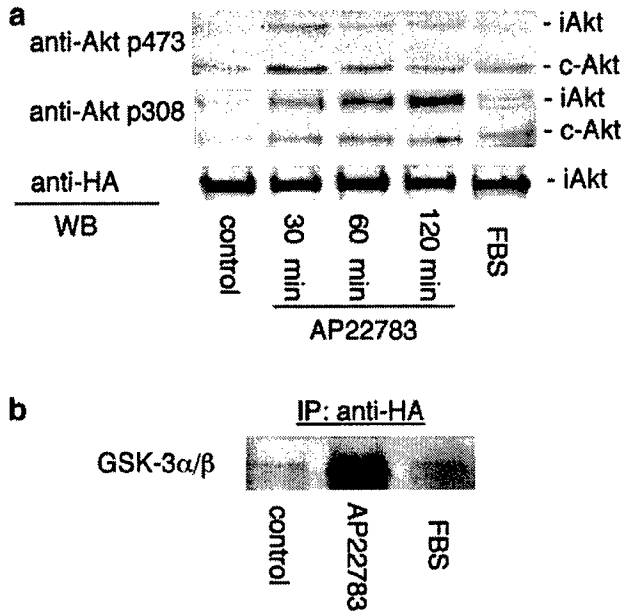

Figure 5 Phosphorylation and activation of iAkt following CID-mediated membrane targeting. (a) 293T cells were cotransfected with M-FRB$_1$2 plus F3-ΔPH.Akt followed by 24 h of serum starvation. Thereafter, cells were untreated (negative control), treated with FBS for 120 min (positive control) or AP22783 for 30, 60 or 120 min. Western blot using anti-phospho-specific antibodies against Akt T308 or S473 measured the extent of phosphorylation of endogenous Akt or iAkt. Anti-HA blotting served as a loading control. c-Akt, endogenous Akt. (b) Jurkat.iAkt cells were serum-starved for 24 h followed by treatment with AP22783 or serum for 30 min. iAkt was immunoprecipitated from cell lysates using anti-HA antibody and incubated with the GSK-3 'crosstide' as a substrate. Phosphorylated GSK3 crosstides were quantified by SDS-PAGE separation and immunoblotting with a phospho-specific antibody against GSK-3α21/β9.

CID-mediated membrane targeting of iAkt results in PI3K-independent activation

For maximum utility, an ideal CID-inducible protein would respond only to CID, but not to environmental signals, such as growth factors. Since endogenous Akt is a major effector molecule of PI3K signaling,[6] inhibition of PI3K leads to inhibition of c-Akt. However, the activity of membrane targeted Akt, such as M-Akt, is largely PI3K independent, presumably because basal levels of membrane-associated PDK1 are sufficient for Akt phosphorylation. To determine if CID-mediated Akt activation is also largely PI3K independent, the effects of PI3K inhibition, using two different inhibitors, were studied in the NF-κB reporter assay described above. Jurkat-TAg cells were cotransfected with reporter plasmid, NF-κB/SEAP, along with the bicistronic plasmid iAkt$_b$. After 24 h, cells were pretreated with two different concentrations of either wortmannin (1 μM and 10 μM) or LY294002 (5 μM and 50 μM) for 40 min, and then cell aliquots were stimulated with 5 ng/ml PMA and log dilutions of AP22783. SEAP activity was measured 24 h later. As shown in Figure 6a and b, the inhibitors at either concentration did not prevent NF-κB induction by iAkt, although maximal NF-κB activation was moderately effected.

To further compare inhibition of endogenous Akt with iAkt, Jurkat.iAkt cells were serum starved for 24 h followed by 30 min pretreatment with PI3K inhibitors, wortmannin and LY294002, or MEK inhibitor, PD98059, as a control. After inhibitor pretreatment, AP22783 was added to the media for another 30 min to mobilize membrane recruitment of iAkt. As shown in Figure 6c, addition of either PI3K inhibitor significantly blocked endogenous Akt phosphorylation at T308, but had a much smaller, if any (for 1 μM wortmannin), effect on iAkt phosphorylation. The MAPK signaling inhibitor, PD98059, had no discernable effect on either endogenous or iAkt. These results, together with the NF-κB/SEAP assay (Figure 6a, b), indicate that CID-mediated iAkt activation is primarily independent of environmental signaling.

CID-mediated activation of iAkt leads to apoptosis resistance following multiple pro-apoptotic signals

When overexpressed, wild-type or constitutively active Akt has been demonstrated to protect cells from a variety of apoptotic stimuli, including treatment with DNA-damaging agents, PI3K inhibitors, Fas-crosslinking, UV (or γ) irradiation, c-myc overexpression, growth factor withdrawal, TGFβ treatment, matrix detachment, or cell cycle perturbation (reviewed in Ref. 5). To test whether CID-mediated activation of chimeric Akt could also protect cells from apoptosis, Jurkat.iAkt cells were given various apoptotic insults in the presence or absence of rapalogs.

Initially, we assessed the protective effects of iAkt on staurosporine (STS)-induced apoptosis. Although staurosporine is a potent inhibitor (IC_{50} ~3 nM) of many PKC family members,[47] it can also inhibit other S/T and tyrosine kinases at higher concentration.[48] Therefore, the exact mechanism of triggering apoptosis is likely to be complex. Following serum starvation for 24 h, Jurkat.iAkt cells were treated with 0, 0.5 or 2.0 μM STS with or without AP22783 (400 nM) for 6 h in the absence of serum. Cell death was monitored using propidium iodide (PI) staining and flow cytometry (FCM) by quantitation of subdiploid cells. As expected, STS induced cell death in a dose–response manner (Figure 7a). At low-dose STS treatment, 19.4% of cells were apoptotic after 6 h, but this toxicity could be fully blocked by AP22783 treatment. Further, although high-dose STS treatment triggered greater apoptosis, iAkt activation was able to prevent, or delay, apoptosis for the majority (36% to 17% without background correction) of 2 μM STS-treated cells.

To further confirm the anti-apoptotic effect of iAkt activation on STS-induced cell death, caspase-3 activation and PARP substrate cleavage were measured by immunoblotting. As shown in Figure 7b, STS treatment at low- and high-dose resulted in dramatic PARP cleavage and significant reduction of procaspase-3 (evidence for procaspase-3 activation) at high-dose. However, AP22783 addition blocked STS-induced caspase-3 activation and low-dose STS-induced PARP cleavage, and also reduced high-dose STS-induced PARP cleavage. Thus, activation of iAkt can block or, at minimum, delay apoptosis by the potent apoptosis inducer, STS.

As shown in Figure 6, CID-mediated activation of chimeric ΔPH.Akt and induction of NF-κB are largely independent of PI3K. To further characterize the ability of iAkt activation to block apoptosis, we directly examined the effects of AP22783 treatment following PI3K inhibition of Jurkat.iAkt cells. Serum-starved Jurkat.iAkt cells were treated with half-log dilutions of wortmannin (0.03–10 μM) or LY294002 (0.3–100 μM) with or without AP22783 (400 nM) in low (2%) or high (10%) FBS-containing medium for 9 h (Figure 8a and b and not shown). In the presence of either low or high levels of FBS, the PI3K

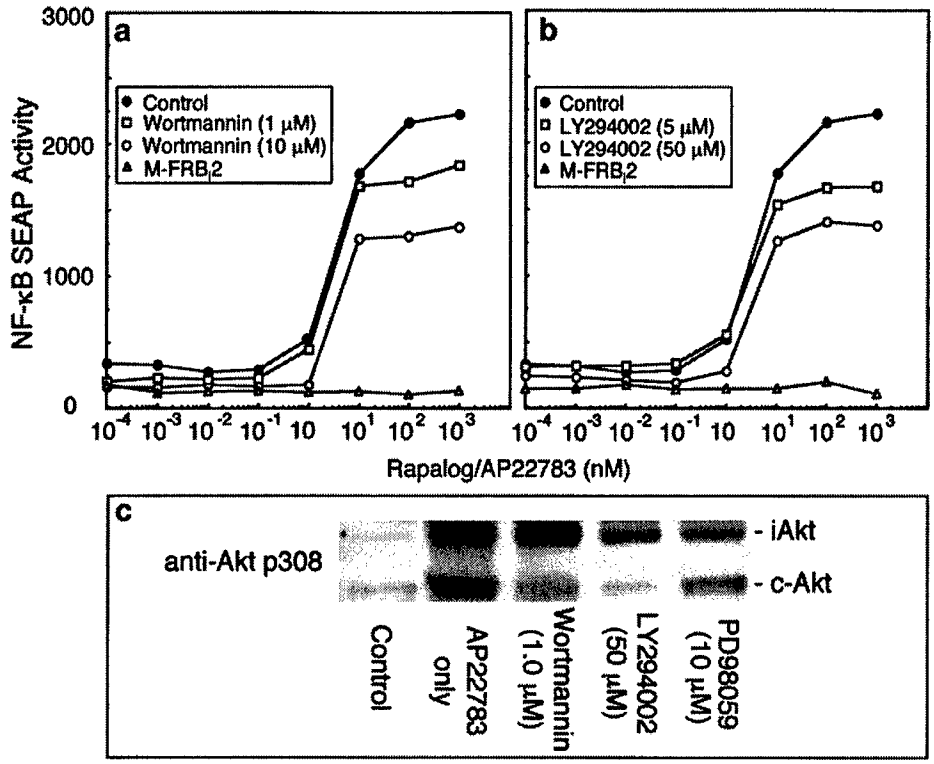

Figure 6 NF-κB transactivation induced by CID-mediated iAkt activation is PI3K independent. (a and b) Jurkat-TAg cells were transfected with bicistronic construct iAkt$_b$ and the NF-κB/SEAP reporter. Cells transfected with M-FRB$_L$2 served as a negative control. After 24 h, cells were treated with half-log dilutions of AP22783 in PMA (5 ng/ml)-containing media plus or minus PI3K inhibitors, wortmannin (a) or LY294002 (b) at two concentrations. SEAP activity was measured 24 h later, and data are representative of two experiments. (c) PI3K inhibitors prevent activation of c-Akt, but not iAkt. Following serum starvation for 24 h, Jurkat.iAkt cells were pretreated for 30 min with PI3K inhibitors, wortmannin (1 μM) or LY294002 (50 μM), or the MEK inhibitor PD98059 (10 μM) followed by incubation with AP22783 (400 nM) for an additional 30 min. Akt phosphorylation was determined by immunoblotting with phospho-specific antibody against Akt/T308.

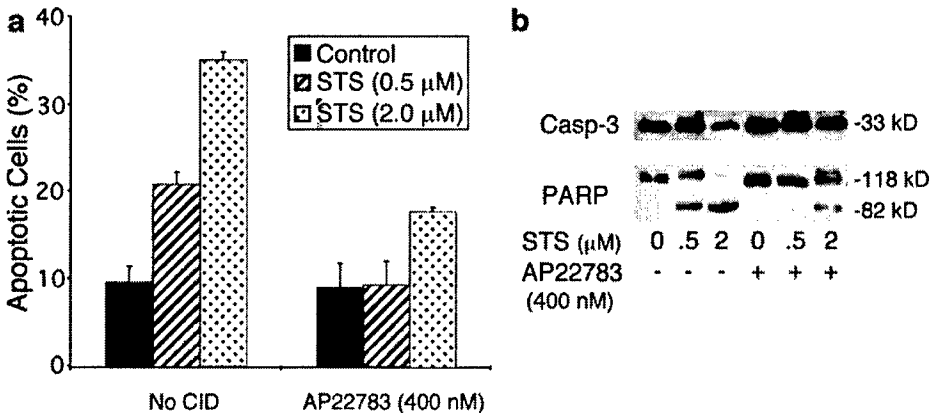

Figure 7 CID-mediated activation of Akt kinase blocks staurosporine (STS)-induced caspase-3 activation, PARP cleavage, and apoptosis. (a) Following serum starvation for 24 h, Jurkat.iAkt cells were treated with STS (0.5 or 2.0 μM) with or without (control) AP22783 (400 nM) for 6 h in serum-free conditions. Hypodiploid/apoptotic cells were determined by flow cytometry after PI staining of permeabilized cells. (b) Following serum starvation for 24 h, Jurkat.iAkt cells were treated with different doses of STS (0.5 and 2.0 μM) with or without AP22783 for 6 h in serum-free condition. Caspase-3 activation was determined by Western blotting using anti-caspase-3 antibodies as a decrease in inactive pro-caspase-3, and PARP (full-length and processed) was detected using anti-PARP antibodies.

inhibitors induced increasing cell death in a very clear dose-dependent manner. In the presence of AP22783, however, cell death was almost totally blocked regardless of the FBS concentration. These results further demonstrate that CID-mediated Akt activation can mitigate PI3K inhibition-induced cell death.

It is well established that oligomerization of the Fas receptor can result in rapid formation of the cytoplasmic death-inducing signaling complex (DISC) and activation of a caspase cascade that leads to apoptosis. Recent data have revealed that activation of the PI3K-Akt pathway can protect cells from Fas-mediated death.[49,50] Therefore,

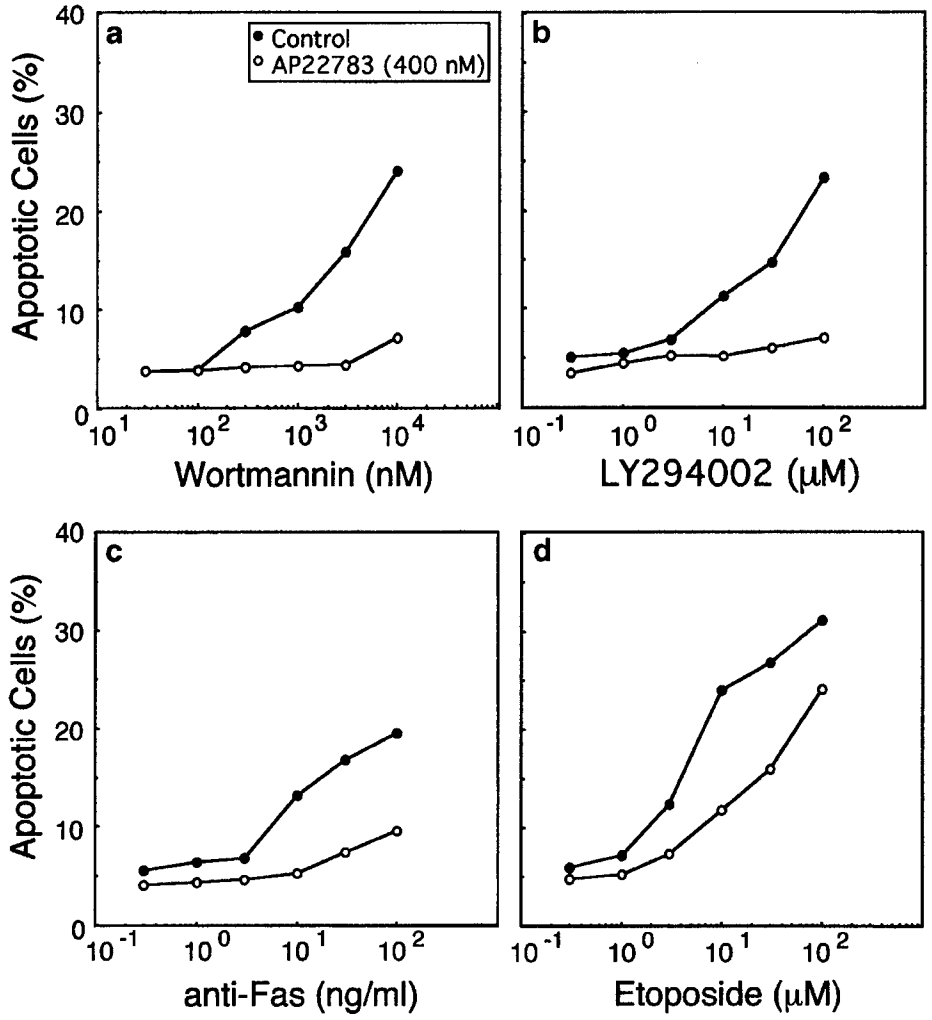

Figure 8 *CID-mediated activation of Akt kinase blocks apoptosis triggered by multiple stimuli. Following serum starvation for 24 h, Jurkat.iAkt cells were treated with half-log dilutions of (a) wortmannin (0.03–10 μM), (b) LY294002 (0.3–100 μM), (c) anti-Fas antibody, CH-11 (0.3–100 ng/ml), or (d) etoposide (0.3–100 μM) with or without AP22783 (400 nM) for 6 (c, d) or 9 (a, b) h in 10% FCS media. Apoptotic cells were measured by flow cytometry for subdiploid populations after PI staining.*

to further investigate the potential utility of iAkt activation, we tested the effect of CID-mediated Akt activation on Fas-induced apoptosis in Jurkat.iAkt cells. After 24 h serum starvation, cells were treated with half-log dilutions (0.3–100 ng/ml) of the anti-Fas antibody, CH-11, in the presence or absence of AP22783 (400 nM) in low (2%) or high (10%) FBS for 6 h, as above. As shown in Figure 8c, Fas receptor engagement induced by CH-11 resulted in apoptosis in a dose-dependent fashion in both low (not shown) and high FBS levels. However, regardless of the FBS concentration, AP22783 treatment rescued cells from CH-11-triggered apoptosis, indicating that the synthetic activation of iAkt is also able to partially protect cells from the deleterious effects of Fas signaling.

Finally, we examined the anti-apoptotic effects of iAkt on protection from direct DNA damaging agents. The antitumor agent, etoposide (VP16), is widely used as a chemotherapeutic drug that works by inhibiting DNA topoisomerase II, which can induce apoptosis in a variety of cell types (reviewed in Ref. 51). To test if etoposide-induced cell death can be blocked by CID-mediated activation of Akt kinase, Jurkat.iAkt cells were serum-starved for 24 h followed by treatment with half-log dilutions of etoposide (0.3–100 μM) with or without AP22783 (400 nM) for 12 h. Again, the experiments were carried out in two different culture conditions with low (not shown) or high FBS levels. As shown in Figure 8d, AP22783 addition reduced etoposide-induced cell death efficiently in high FBS conditions. A lesser, but reproducible level of protection was seen under low FBS conditions (not shown). Although the ability of iAkt to delay or block apoptosis following etoposide treatment was not as extensive as blocking apoptosis triggered by STS, PI3K inhibition or Fas ligation, these results demonstrate that the CID-activated Akt can be a powerful 'live switch' to prevent, or delay apoptosis by multiple stimuli.

Discussion

In mammals, multiple signaling molecules are focused on triggering or inhibiting apoptosis, underscoring the importance of rapid altruistic cellular suicide after obsolescence, extensive DNA damage, viral infection, or other stresses. For example, recent reports from the genome

project suggest that in humans there are at least 14 caspases, 11 Bcl-2 family members, 30 death domain-, seven death effector domain-, 20 CARD domain-, and eight BIR domain-containing proteins. Following activation by extracellular growth/survival factors, Akt appears to be a nodal upstream signaling molecule involved in inhibiting, or at least delaying apoptosis triggered by diverse stimuli. With this in mind, we developed a conditional allele of Akt, called iAkt, which can be activated following administration of a small, lipid-permeable ligand related to rapamycin.

The development of iAkt takes advantage of the mounting evidence that induced proximity is a fundamental feature of cell signaling. In order to increase the efficiency of an enzymatic reaction, enzymes, such as kinases and phosphatases are localized to regions of the plasma membrane (and/or internal membranes) along with their substrates. These interactions are typically organized by adaptor/scaffolding molecules or by membrane-targeting domains, such as the PH domain (reviewed in Refs 52 and 53). Unlike myristoylation (Myr) or prenylation-targeting CAAX domains (found on many G proteins), PH domains lead to primarily regulated membrane localization based on the PI3K-mediated accumulation of D3-phosphorylated PtdIns compounds. Using CID-mediated membrane targeting, however, all three domains can be replaced with CID-binding domains for artificial regulation. This approach to conditional regulation of cytosolic signaling proteins was first demonstrated for Src family kinases, and G-proteins using the nonspecific homodimeric CID, FK1012, that binds equally well to endogenous and ectopically introduced FKBP12 domains.[25] In contrast, rapalogs bind specifically to FRB_1, permitting more exact control of heterodimerization. Furthermore, the compact size of rapalogs has desirable consequences with regards to solubility, biodistribution and membrane permeability.

Since the primary Akt kinase, PDK1, is also recruited to the plasma membrane via its PH domain, it appears somewhat unexpected that CID-activated iAkt, or even Myr-Akt alleles, should be insensitive to PI3K activation. Nevertheless, most reports suggest that Myr-Akt is largely PI3K independent. One likely explanation is that the basal level of membrane-associated PDK1 is low, but high enough for stochastic interactions with membrane-localized Akt and phosphorylation of T308 to accumulate. Once activated, Akt is ultimately down-regulated due to normal homeostatic feedback mechanisms that include reducing D-3-phosphorylated PtdIns.[17] This mode of regulation should be circumvented by the removal of the Akt PH domain along with a second function, inhibition of access to T308 (reviewed by Ref. 5). The PH domain has also been associated with multimerization of Akt, which may be important for auto-phosphorylation at S473.[54] However, membrane localization, even in the absence of direct crosslinking, may evoke a threshold level of stochastic Akt interactions for the accumulation of S473 phosphorylation and hence full activation.

The other fortuitous surprise was the extremely low background of iAkt. Initial attempts by ourselves and others to design a conditional Akt using CID technology failed, probably due to the high basal activity of overexpressed full-length Akt (Figure 3a) or the inefficiency of rapamycin-based heterodimerization combined with any biological activities of rapamycin at the doses used.[55] Interestingly, Roth and colleagues were able to design a 4-hydroxytamoxifen-inducible Akt by fusing a constitutively active Myr-ΔPH.Akt to a mutant hormone-binding domain from the estrogen receptor.[55] The availability of two distinct approaches to make conditional signaling molecules should permit independent activation of Akt and other signaling molecules in the same cell.

Although a number of applications of CID technology have been reported using high-specificity homodimerizing CIDs (CID_{hod}), or heterodimerization with bioactive rapamycin, this study represents the first demonstration of regulating a cytoplasmic signaling molecule with bioinert CID_{hed} first described for use in activating transcription.[56] This new application should also be useful for activating multiple signaling molecules that are normally regulated by membrane localization. This includes not only proteins with membrane targeting, PH, myristoylation, or prenylation (eg CAAX) domains, but also proteins that are recruited to other membrane-localized molecules via protein–protein interaction domains, such as SH2, PTB, or SH3 domains. For example, we have also made highly inducible Src family kinases (eg Fyn, Lck) by replacing their myristoylation-targeting domains with FKBP12 and recruiting these chimeric proteins to M-$FRB_1$2 using rapalogs (unpublished results).

The development of iAkt and other inducible signaling molecules should have broad utility. The low basal activity and rapid induction of Akt should permit genome-wide identification of Akt substrates that might be otherwise down-regulated, dephosphorylated, or indirectly phosphorylated in cells carrying constitutively active Akt alleles. Further, since Akt is often up-regulated in various tumors, including breast, ovarian and prostate, and v-Akt is an oncogene, transgenic models bearing tissue-specific inducible Akt should lead to multiple neoplastic models.[57] Further, regulating the survival of gene-modified cells following transplantation with an Akt-based 'survival switch' may complement the use of suicide genes,[58,59] permitting an additional dimension of control for gene therapy.

Considerable attention has focused recently on protection of multiple (eg brain, liver, heart) tissues from damage that follows ischemia and reperfusion. Many of these efforts are focused on the use of viral vectors to transfer anti-apoptotic genes. While overexpression of Bcl-2 has been the most commonly used gene for this purpose,[60–64] constitutively active Myr-Akt has also been employed to protect animals from injury, such as transient cardiac ischemia.[65] Similarly, hyperoxia-induced apoptosis can also be reduced using Myr-Akt.[66] Further, several groups have shown that tissue grafts or gene-modified target tissue may better be able to withstand cellular and humoral immunity following transfer of Bcl-2.[67–70] While these previous studies clearly demonstrate the potential utility of preventing apoptosis during transient injury or immunogenic conditions, longer longitudinal studies are likely to temper the long-term safety prospects of overexpressing constitutively active oncogenes *in vivo*. A reversible 'anti-death switch', such as iAkt, should prove to be a safer alternative.

Materials and methods

Plasmids construction

To generate F3-Akt, F3-ΔPH.Akt, F3-AktKM and variants, Akt and ΔPH.Akt were *Pfu*I (Stratagene, La Jolla, CA, USA)-amplified from pCMV6-HA-Akt[71] or pCMV6-HA-Akt K179M[72] using *Sal*I-linkered 5′ primers, mAkt5SPH (full-length): 5′-aga gcgac aac gac gta gcc att gtg aag gag-3′ or mAkt5S (truncated ΔPH): 5′-aga gtcgac acc gcc att cag act gtg gcc-3′ and 3′ primer, mAkt3S: 5′-aga gtcgac ggc tgt gcc act ggc tga gta g-3′. PCR products were subcloned into pCR-Blunt (Invitrogen) or pKSII$^+$ (Stratagene) and sequence verified, to create pSH5/mAkt, pSH5/mΔPH.Akt, and pKS/mAkt.KM. The 1440-bp full-length Akt and 1130-bp ΔPH.Akt fragments were removed with *Sal*I and subcloned into *Xho*I/*Sal*I-digested M-F_{pk} 3-E, or *Xho*I or *Sal*I-digested S-F_{pk} 3-E, described previously,[30] to create M-Akt (and Akt variants), Akt-F3 (and variants) and F3-Akt (and variants). Note, all chimeric proteins contain the HA epitope (E), but the 'E' is left off (along with 'pk' subscripts) for simplicity. To generate myristoylated rapalogue-binding domains, the rapamycin binding domain (FRB) from human FRAP (res. 2025–2113; T2098L) was *Pfu*I-amplified from FRAP*-AD[35] using primers, 5FRBX: 5′-cgat ctcgag gagatgtggcatgaaggcctgg-3′ and 3FRBS: 5′-cgat gtcgac ctttgagattcgtcggaacacatg-3′ and subcloned into pCR-Blunt to produce pSH5/FRB_1. One or two copies of the *Xho*I/*Sal*I FRB_1 domain were subcloned into *Xho*I/*Sal*I-digested M-F_{pk} 3-E to create M-FRB_1 and M-$FRB_1$2. The NF-κB-SEAP reporter plasmid was described previously.[25]

To make the bicistronic iAkt constructs, two different internal ribosome entry sequence (IRES) elements from EMCV or poliovirus were used to link M-$FRB_1$2 and F3-ΔPH.AKT on the same transcript. The poliovirus IRES sequence (IRESp) was *Pfu*I-amplified from pTPOV-3816[73] with primers, 5pIRES/Mn: 5′-ata caattg ccgcgg ttc gaattc tgttttatactcccttcccgtaac-3′ and 3pIRES/Mun; 5′-tat caattg gtttaaac agcaaacagatagataatgagtctcac-3′. The resulting PCR products were subcloned into pCR-Blunt to create pSH5/IRESp-Mun. The 615-bp IRESp *Mun*I fragment was ligated into *Eco*RI-digested pSH1/M-$FRB_1$2-E to create pSH1/M-$FRB_1$2-E-IRESp. Finally, the *Not*I/*Eco*R1 F3-ΔPH.AKT fragment from pSH1/F3-ΔPH.AKT was blunt-ligated into the *Pme*I site to create pSH1/M-$FRB_1$2-E-IRESp-F3ΔPH.Akt, renamed as $iAkt_b$. The bicistronic vector $iAKt_a$ utilizes the EMCV IRES and was made by a comparable strategy. For establishing Jurkat.iAkt cell lines, the bicistronic *Not*I/*Mun*I fragment from $iAkt_b$ was subcloned into *Not*I/*Eco*RI-digested pBJ5-neo (described previously) to create pBJ5-neo/$iAkt_b$.

Cell culture

293T human embryonic kidney cells (ATCC, Manassas, VA, USA) and Jurkat (ATCC), Jurkat-TAg[74] and Jurkat.iAkt were maintained in DMEM or RPMI-1640, respectively, containing 10% fetal bovine serum (FBS) and antibiotics. The Jurkat.iAkt line was derived by transfecting Jurkat cells with *Nde*I-linearized pBJ5-neo/$iAkt_b$ plasmid followed by G418 (1 mg/ml) selection. Clones were screened by anti-HA immunoblotting.

Reagents

Etoposide, propidium iodide, RNase A and staurosporine were from Sigma (St Louis, MO, USA); wortmannin and LY294002 were from Calbiochem (La Jolla, CA, USA); Rapalog/AP22783 was from Ariad Pharmaceuticals (Cambridge, MA, USA; www.ariad.com/regulationkits/). Anti-Fas antibody (CH.11) was obtained from Kamiya Biomedical (Thousand Oaks, CA, USA), monoclonal anti-HA (HA.11) was from BAbCO (now CRP, Princeton, NJ, USA), polyclonal anti-HA was from Upstate Biotechnology (Lake Placid, NY, USA), anti-caspase-3 was from Santa-Cruz Biotechnology (Santa Cruz, CA, USA) and anti-PARP was from Roche-Boehringer-Mannheim (Indianapolis, In, USA).

Electroporation and SEAP assay

Jurkat-TAg cells in logarithmic-phase growth were electroporated (950 μF, 250 V; Gene Pulser II (BioRad, Hercules, CA, USA)) with expression plasmids and 2 μg of the NF-κB-SEAP reporter plasmid. After 24 h, transfected cells were stimulated with sub-optimal levels of the phorbol ester PMA (5 ng/ml) along with log dilutions of the heterodimerizing CID, AP22783, and additional treatments as stated in the figure legends. After an additional 24 h, supernatants were assayed for SEAP activity as described previously.[25]

Western blots

293T cells seeded in six-well plates were transiently transfected with 2 μg of different expression constructs in 6 μl FuGENE6 (Boehringer-Mannheim, Indianapolis, In, USA) in Opti-MEM-I medium for 24 h followed by serum starvation for an additional 24 h. Cells were then treated with different agents and harvested at different time-points as stated. After washing twice in ice-cold PBS, cell pellets were lysed in RIPA buffer containing protease inhibitors (CytoSignal, Irvine, CA, USA). Equal amounts of protein from each sample were separated on 10% SDS-PAGE gels and transferred to PVDF membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Phospho-specific antibodies against Akt (T308 or S473 site) (Cell Signaling, Beverly, MA, USA) were used for measuring Akt phosphorylation, and the signal was detected by AP-conjugated secondary antibodies (NEB, Beverly, MA, USA) and CDP-Star chemiluminescence reagent (NEN Life Science, Boston, MA, USA).

Immunoprecipitation and in vitro Akt kinase assay

Jurkat.$iAKt_b$ were serum starved for 24 h followed by treatment with AP22783 or serum for 30 min. Cells were then lysed in a lysis buffer provided with the Akt Kinase Assay kit (Cell Signaling, Beverly, MA, USA), and F3-ΔPH.AKT-E was immunoprecipitated with polyclonal anti-HA antibody. Antibody–antigen complexes were washed three times in lysis buffer and once in kinase buffer. *In vitro* kinase assays for Akt were performed using a GSK3α/β 'crosstide'. The extent of crosstide phosphorylation was determined by anti-GSKα/β immunoblotting according to the manufacturer's protocol.

Apoptosis and flow cytometry

Jurkat.iAkt were serum starved for 24 h followed by pretreatment with AP22783 in 0, 2 or 10% FBS for 40 min. After incubation with apoptosis-inducing stimuli for the periods indicated, cells were harvested and washed twice in ice-cold PBS and fixed in 70% ethanol. Cells were stained in 50 μg/ml propidium iodide and 100 μg/ml RNase A for 30 min at 37°C, and hypodiploid cells were

quantitated by flow cytometry using a Beckman-Coulter EPICS XL-MCL. For determination of caspase-3 activation and PARP cleavage after staurosporine treatment, cell pellets were lysed in Laemmli sample buffer containing 5% (v/v) β-mercaptoethanol (Bio-Rad, Hercules, CA, USA), and equal amounts of protein were separated on 6 (for PARP) or 12% (for caspase3) SDS-PAGE followed by immunoblotting with anti-caspase-3 and anti-PARP antibodies.

Note added in proof

The high basal activity of F3-Akt and the observed PI-3K-independence of iAkt signaling may be partly explained by the high basal levels of phosphatidylinositides in Jurkat cells and other tumor lines, due to PTEN deficiency.[75]

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